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Study of Outer Membrane Vesicles Isolated from *B. abortus* S19 by Bulk Production and Characterization

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A B S T R A C T

Outer membrane vesicles (OMV) are closed spheroid vesicles with size ranging between 20–500 nm produced by both Gram negative and Gram positive bacteria. OMV are formed by the blebbing and pinching off segments of bacterial outer membrane. They are composed of lipopolysaccharide (LPS), glycerophospholipids, outer membrane proteins (OMPs), and periplasmic components. They are found in a variety of environments including liquid culture, solid culture and biofilms as well as during periods of bacterial stress. Being small size and having immunogenic properties and non-replicative behavior they stand as a good choice for vaccination. Keeping in view of the above facts the present study has been carried out to explore the immunogenic properties of OMV by its isolation and characterization. Isolation of OMV from *Brucella abortus* S19 was carried out by growing in solid as well as liquid media and subsequently the growth was subjected to filtration and centrifugation steps. The OMV were characterized by electron microscopy. They were further analyzed by SDS-PAGE and Western Blotting. On electrophorectic profile they revealed protein bands of 86 kDa, 70Kda, 65kDa 40 kDa and immunore active band of 86, 70, 65 kDa on western blotting.

Introduction

One of the most important bacterial diseases worldwide brucellosis has been classified by World Health Organization as world leading neglected zoonotic disease (OIE, 2010). It severely hinders livestock productivity and human health worldwide. *Brucella abortus* is the main causative organism in bovine. Bovine brucellosis has been reported in virtually all countries where cattle are farmed. The disease is endemic in India, a country that house the world's largest cattle and buffalo population and produce the most milk in the world.

Live vaccines like *B. abortus* strain 19 and *B. abortus* RB51 are the most commonly used vaccines for the control of brucellosis in various parts of the world. But they suffer

from some inherent disadvantages of residual abortifacient activity in pregnant animals besides biosafety issues and also cannot be used in the males (Rose *et al*., 2018).

Bacterial pathogens have evolved various mechanisms to transport virulence factors to the eukaryotic host cells. This virulence factors aids in the colonization, immune-evasion, nutrient acquisition and other cell-cell communication thus plays a major role in establishing successful host pathogen relationship.

The release of outer membrane vesicles (OMV) is one such important phenomenon that can disseminate bacterial products and interact with the environment. OMV also known as blebs enable bacteria to secrete insoluble molecules as well as soluble material attached to it and allow it to reach the target site in a concentrated, protected and targeted form.

The vesicles are produced spontaneously and during the normal growth of the bacteria (Beveridge, 1999) which can be found in different environments like soil, biofilms, and enriched culture medium and during the infective process of pathogens.

They play important biological functions on the environment and on other cells by playing an active part in pathogenesis, quorum sensing, horizontal gene transfer (Yaron, 2000). Like other Gram negative bacteria, *Brucella* also releases OMVs to the external environment (Boigegrain *et al*., 2004).

Purification of OMV relies on their small size and buoyant density, allowing them to be separated from the bacterial cells by centrifugation or ultrafiltration (Kuehn and Kesty, 2005). Earliest work done can be date back in 1989 by Gamazo and his co-workers who worked on outer membrane blebs of *B. melitensis* and studied the protein profile. Various authors tried growing the vesicles in solid as well as liquid media. However, they reported the yield in solid media yielded more vesicles as compared to liquid media.

Materials and Methods

Bacterial strains and growth conditions

The *Brucella abortus* S19 used in this study was procured from the Department of Veterinary Microbiology, GADVASU, Ludhiana. The culture was grown in Brucella selective medium (BSM) prepared in Petri-plates with Brucella growth supplements and incubated at 37°C for 48 h.

Preparation of OMV

OMV were isolated from *B. abortus* S19 by using a procedure discussed previously (Gamazo *et al*., 1989). The *Brucella abortus* S19 cultures were grown on a Roux flasks and Petri-plates containing BSM and incubated for 2-3 days at 37°C. When sufficient and enough growth had obtained the culture was harvested with PBS pH 7.4.

The bacterial suspension was centrifuged at 10,000×g for 30 min. The supernatant was filtered with 0.22 µ syringe filter. The filtered supernatant was centrifuged at 100,000 ×g for 2 h at 4°C using Optima XPN-100 Ultracentrifuge. OMV pellet was re-suspended in 100 µl of sterile PBS. OMVs samples were divided into aliquots and stored at a temperature of -80°C until use.

Electron microscope –negative stains

The OMV sample was fixed in 0.5% glutaraldehyde and kept for incubation for 1h at 4°C. 10µl of the OMV sample were allowed to adsorb onto copper coated grid for 5-10 min followed by staining with

phosphotoungstic acid (2%). The grid was dried and was examined in electron microscope (Hitachi H-7650).

Protein assay

Protein content was determined by Bi-cinchoninic acid (BCA) method described by He (2011) using bovine serum albumin as standard.

Sodium dodecylsulphate –polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (Biorad) analysis was carried out as per Laemmli (1970) with slight modification (Laemmli, 1970). The gel casting platforms were assembled and sealed. The stacking (5%) and separating gels (12%) were prepared. A volume of 30µl of OMVs containing 90µg of the protein was mixed with an equal volume of 2X sample loading buffer. The gel was stained with Coomassie brilliant blue (CBB).

Western blotting

The OMV obtained from *B. abortus* cells were subjected to immunoblot analysis. Proteins were transferred from polyacrylamide gels to nitrocellulose membrane and incubated with mouse immune sera directed against OMV. The immunochemical detection was performed using goat anti-mouse IgG- HRP.

Results and Discussion

OMV have been explored as a vaccine candidate due to its immunomodulatory role and unique delivery systems. Several licensed vaccines are based on OMV (Jackson *et al*., 2009). OMV encase various immunogens and appear to be safe as vaccines (Granoff, 2010). Vesicle components like LPS and OM porins acts as pathogen associated molecular patterns (PAMPs) that are presented to the first line of immune system. They are recognized by pattern recognition receptors (PRRs) like toll-like receptors (TLRs) which produce inflammatory response in association with the complement system (Amano *et al*., 2010).

The *B. abortus* culture inoculated in solid media Brucella Selective Medium (BSM) prepared in Roux flasks and Petri-plates were used to obtained OMV. The growth was harvested in PBS and bacterial harvest was subjected to filtration steps. The bacteria free filtrate (BFF) were subjected to ultracentrifugation (1,00,000 × g for 2 h at 4°C) which is one the significant step in the procurement of vesicles. The vesicles were seen as pellet at the bottom of the tube (Fig 1). The pellets were of transparent gel like consistency. After the supernatant has been discarded carefully the pellet were washed with PBS (pH 7.4) in sterile eppendorf and stored at -80°C till its further use. Protease inhibitor cocktail was used to prevent protein degradation. Liquid medium was tried to procure the vesicles but the yield was comparatively less as compared to that from solid medium.

Previous studies were done on *B. ovis* and *B. melitensis* where the outer membrane blebs were extracted by growing on agar plates which were suspended in sterile phosphate-buffered saline. They demonstrated that *B. ovis* also produces blebs on solid media and in liquid media. However, the yield on solid media produced better results with larger size (Gamazo and Moriyon, 1987).

Negatively stained OMV sample showed multiple spherical vesicles with electron dense centre. The size of the vesicles ranged from 150-250 nm (Fig 2). The results were in conformity with the general size of OMVs as reported in Gram negative bacteria (Beveridge, 1999). Further the vesicles were

found to form clumps and aggregates when observed under the microscopy. The protein profiles of OMVs were checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gel. Denaturing gels stained with Coomassie blue allowed for visualization of the protein profile present in the OMV. Protein sizes of 40 kDa, 65kDa, 70kDa and 86 kDa were observed (Fig 3). SDS-PAGE analysis of the free membranous material (FMM) and Sarkosyl-Zwittergent cell envelope (CE) revealed that both materials contained two major proteins (30kDa and 25kDa) and several minor protein bands (i.e. 18, 22, and 84 kDa) with similar apparent molecular weights (Gamazo and Moriyon, 1987).

Previous authors had reported the presence of 88, 66, 26, 23, 18 and 10 kDa sized bands from OMVs of *B. melitensis* (Avila-Calderón *et al*., 2012). Two major bands (25kDa and 30 kDa) and several minor bands (18, 22, and 84 kDa) in the OMVs of both smooth *B. melitensis* 16M and a rough strain *B. melitensis* B115 has also been reported (Gamazo *et al*., 1989). Differences in electrophoretic profile of OMV exists which may be due to the different strains (Avila-Calderón *et al*., 2012). There is also difference in SDS-PAGE profile between *B. abortus* RB51 and *B. abortus* RB51/SOD (Cassidy, 2010).

Immuno blotting was performed to ensure that *Brucella abortus* strain 19 OMV contain immune reactive proteins. The immune blotting was performed using goat anti-mouse HRP conjugate as secondary antibody. The hyperimmune serum raised against *B. abortus* S19 was used as a source of primary antibody in the study. Serum from mice on day 21st post-immunization was used to study the immunore activity which was immunized with OMV S19.

Minor bands between 65, 70, 86 kDa (Fig 4) were observed in our study which may be of OMPs. Immunogenic proteins of OMVs of size 18 kDa and 40-45 kDa of *B. abortus* strain RB51 were also reported (Cassidy, 2010). Differences in the protein may be due to the different strains of *Brucella* used by other workers.

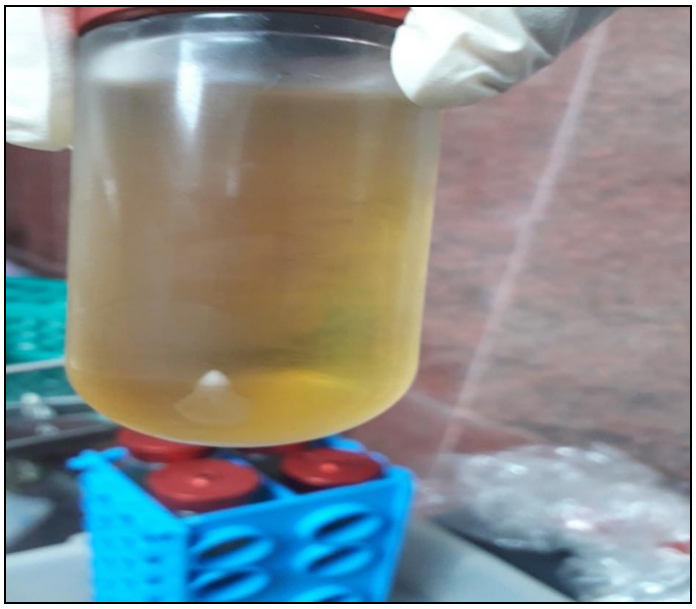

Fig.1 OMV seen as pellet after ultracentrifugation

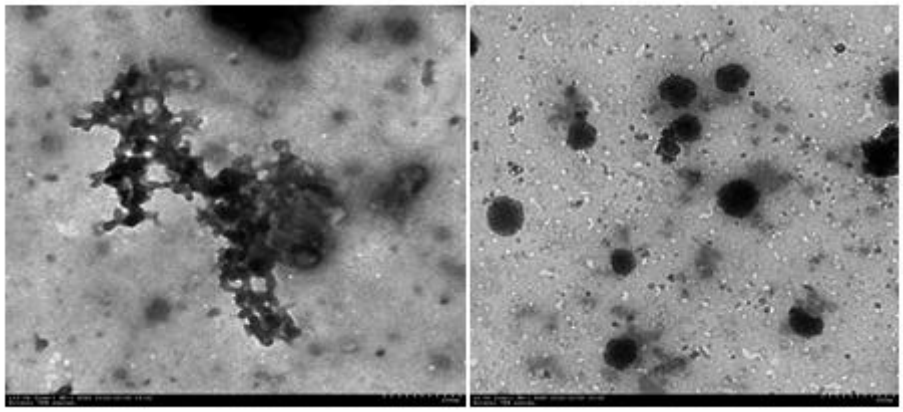

Fig.2 Transmission electron microscopy of OMV of *B. abortus*S19

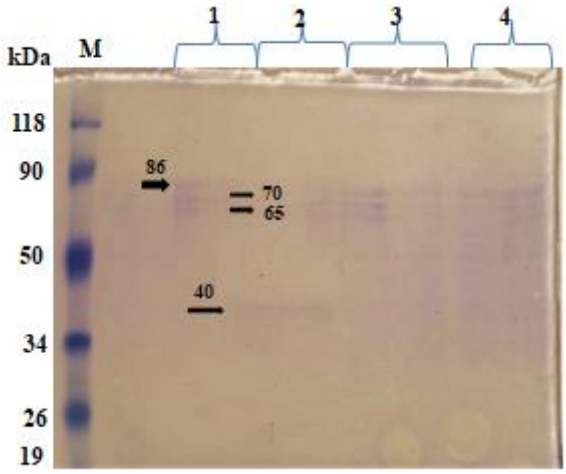

Fig.3 SDS-PAGE analysis of OMV of *B. abortus*S19

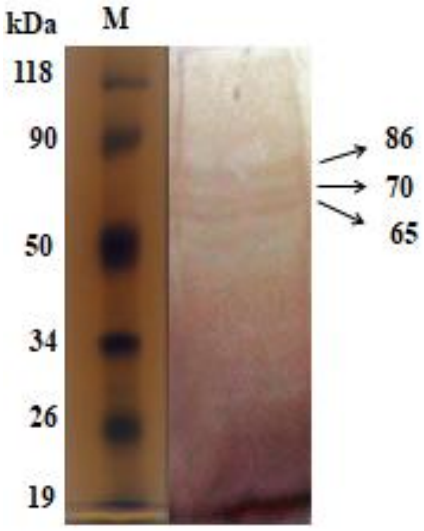

Fig.4 Western blot analysis of OMV of *B. abortus*S19

The development of safe and effective vaccines against both animal and human disease will constitute an important step in curbing brucellosis. Like other Gram negative bacteria, *Brucella* also releases OMVs to the external environment (Boigegrain *et al*., 2010). The use of *Brucella* OMVs as a potential vaccine candidate has also been explored and previous studies has shown to purified OMVs from both *B. melitensis* smooth strains 16M and rough VTRM1 strain by differential centrifugation were used to immunize mice and was found to provide partial protection against direct challenge of *B. melitensis* in mice model (Avila-Calderón *et al*., 2012). This present study has demonstrated that OMV can be isolated and characterized by various methods as described above. Further studies are required to study the potential of OMV to check for the immune response studies and determined the efficacy as a vaccine candidate.

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